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Repeated DNA Sequences and Kangaroo Phylogeny

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Abstract

Three highly repeated DNA sequences have been used to determine relationships of species within the Macropodidae (kangaroos and wallabies). Two highly repeated DNA sequences were isolated as buoyant density satellites in the red-necked wallaby and in the wallaroo–euro group. The third probe was a cloned representative of one class of highly repeated species from the red kangaroo.

Radioactively labelled probes of these three repeated sequences were used to determine the incidence and distribution of each in a number of macropodid species. The results were consistent with a monophyletic origin of the macropodid species and showed in particular, that the red-necked wallaby is closely related to the red kangaroo and to the euro–wallaroo group. In addition, the data indicated that the tammar and the agile wallaby were closely related. The results also favour the current taxonomic status of the eastern and western grey kangaroos as closely related but separate species.

Introduction

The Macropodidae, containing about 60 living species, has always been regarded as a coherent taxonomic group of marsupials. However, while some genera are clear cut, e.g. the rock-wallabies (*Petrogale*) and the tree-kangaroos (*Dendrolagus*), there have been differences of opinion among taxonomists over the placing of a group of 15 species, the typical kangaroos and wallabies, and their relationship to each other. Four generic names have been used for various members of this group during the last twenty years. It is now generally accepted that one species, the swamp wallaby (*Wallabia bicolor*), is distinct on morphological and karyotypic grounds and its placing in a genus of which it is the only living member is warranted (see e.g. Bartholomai 1976). It seems clear also that *Wallabia* has existed as a genus distinct from other Macropodidae since at least late Miocene (Bartholomai 1978). Until recently the red kangaroo (*Macropus rufus*) was usually placed in a monotypic genus *Megaleia* although there was little reason for this beyond a difference in chromosome number, *Megaleia* having $2n = 20$, other marsupials having $2n = 16$, except for the swamp wallaby with $2n = 10, 11$. The remaining species have been variously included in *Macropus*, *Osphranter* or *Wallabia* but all are now usually placed in *Macropus* (Kirsch and Calaby 1977).

The relationships of the *Macropus* species remain obscure in spite of a number of studies using a variety of techniques. These have included chromosome morphology (e.g. Sharman 1973, 1974; Hayman and Martin 1974), G banding (Rofe 1978), tissue protein electrophoresis (e.g. Richardson *et al.* 1973; Richardson and McDermid

1978), and comparative serology (Kirsch 1977). Relationships have also been inferred from the occurrence of interspecific hybrids that are sometimes produced in captivity. A number of such crosses have been studied between various species of *Macropus* and, strangely enough, between *W. bicolor* and the agile wallaby *M. agilis*, and *W. bicolor* and the red-necked wallaby *M. rufogriseus* (Hayman and Martin 1974; Smith *et al.* 1979).

One group of taxa that are closely related, as judged by several criteria, includes the eastern wallaroo (*M. robustus robustus*) and euro (*M. r. erubescens*), two subspecies that freely interbreed, but which occupy distinct geographical areas except for a zone of intergradation in Queensland, and which have distinct X chromosome morphologies (Richardson and Sharman 1976). Other taxa in this group are the isolated Barrow Island euro (*M. r. isabellinus*), the northern euro (*M. r. woodwardi*), the black wallaroo (*M. bernardus*), and the antilopine kangaroo (*M. antilopinus*). The latter two species have distinctive morphological features, and *M. bernardus* also differs in chromosome number; Sharman and Calaby (unpublished data) have determined it as $2n = 18$, whereas all other taxa have $2n = 16$. This group (the euro–wallaroo group) is believed to be close to the red kangaroo. Most other species of *Macropus*, e.g. *M. rufogriseus*, *M. agilis* or *M. parryi* (the whiptail wallaby) are not readily placed with respect to the euro–wallaroo group or to each other. The eastern and western grey kangaroos (*M. giganteus* and *M. fuliginosus*) were once considered to be geographical subspecies or even colour morphs, but are now regarded as separate species (Kirsch and Poole 1972).

Fossil evidence has so far been of little use in attempts to determine relationships in living *Macropus* species. The genus is of relatively recent origin and there was a very rapid radiation during the latter part of the Pliocene and the Pleistocene (Bartholomai 1972, 1978). The taxonomic difficulties are compounded by the fact that there are inconsistent similarities and differences between species and species groups, perhaps as a result of independent retentions and losses of both ancestral and rapidly evolved derived characters. A further problem that must be kept in mind is convergence. Richardson *et al.* (1978) and Richardson and McDermid (1978) have discussed some examples of convergence in the distribution of biochemical characters within the Macropodidae.

In this paper we discuss a further approach, using DNA sequences, that may give evidence on the relatedness of different taxa. When two groups of organisms no longer interbreed, it can be expected that the DNA sequences in their genomes will diverge by mutation, the degree of sequence differentiation being correlated with the length of time that divergence has been maintained.

The extent of sequence similarity can be assayed in nucleic acid hybridization experiments, and, following the advent of recombinant DNA methodology and DNA sequencing techniques, by direct sequence analysis of long tracts of nucleotides. This approach is free of many of the variables introduced to other taxonomic methods by gene interactions and by effects of environment on gene expression. The use of DNA sequences in taxonomy is complicated by the existence of different repetition classes of DNA sequences (Britten and Kohne 1968). The sequence of nucleotides coding for an array of amino acids in a protein is present only once or a very few times in the genome and can provide a direct comparative tool. On the other hand, there are sequences which are repeated many times, with perhaps millions of copies being present.

This highly repeated DNA has an inherent property which enables its repetition frequency to be modified over quite short evolutionary time periods. Consequently, negative results concerning particular highly repeated sequences are of little value in providing information about the relationship of different species of animals or plants. Where sequences are detected in more than one species they can be of use in determining taxonomic relationships. This class of DNA can readily provide probes for assays among related species.

Materials and Methods

Isolation of DNA and preparation of satellite DNAs by Ag^+/Cs_2SO_4 gradients or actinomycin D–CsCl gradients were as described in Dunsmuir (1974) and Venolia and Peacock (1981). The preparation of [^{3}H]cRNA, filter and *in situ* hybridization methods were also as described in Venolia and Peacock (1981). Restriction endonuclease digestions, radioactive labelling of DNA, and transfer of DNA to nitrocellulose filters for hybridization were described in Dennis *et al.* (1980).

For cloning of the EcoRI fragment from the red kangaroo, EcoRI digested red kangaroo DNA was subjected to electrophoresis on an 0·8% agarose gel. The 1·4 kb band was sliced out of the gel and electro-eluted (Weinand *et al.* 1979). 5 μg of this isolated fragment was ligated to 2·5 μg of EcoRI digested pAC184 (Chang and Cohen 1978). *E. coli* strain RRI was transformed and colonies selected by hybridization using the radioactively labelled 1·4 kb band as probe. Sequencing of DNA was by the method of Maxam and Gilbert (1977).

Results and Discussion

Highly Repeated DNA Sequences

Highly repeated sequence DNA is a significant component of the genome of almost all higher organisms, and marsupials are no exception. Because the repeating unit of nucleotides is short, this DNA frequently has a different base composition to that found in the remainder of the genome, and it separates as a 'satellite DNA' from the main band of DNA in buoyant density equilibrium centrifugation. This property means that it can be readily isolated in a highly purified form, and has facilitated the analysis and definition of its major properties, including molecular organization and chromosomal distribution.

We have used three highly repeated DNA sequences to investigate relationships among various species of kangaroos. In part, our results have confirmed conclusions made on the basis of more traditional criteria, but in addition, we have been able to suggest new conclusions concerning the phylogeny of some of the wallabies and kangaroos.

We examined the nuclear DNA of a number of kangaroo species to determine if any satellite DNA bands are present. Analytical centrifuge traces (Fig. 1) show that the red-necked wallaby has a prominent satellite of density greater than that of the main band. The euro, wallaroo and antilopine kangaroo also have a dense satellite, its density (1·710 g/cm^3) being slightly greater than that of the red-necked wallaby satellite (1·708 g/cm^3). The red kangaroo and the whiptail wallaby have asymmetric DNA profiles with a shoulder on the heavy side of the peak, indicative of highly repeated DNA species with densities close to, but distinct from, that of the main band of DNA. The absence of satellite peaks in neutral CsCl gradients does not mean that highly repeated DNA species are not present. Other buoyant density conditions can often reveal 'cryptic' satellites.

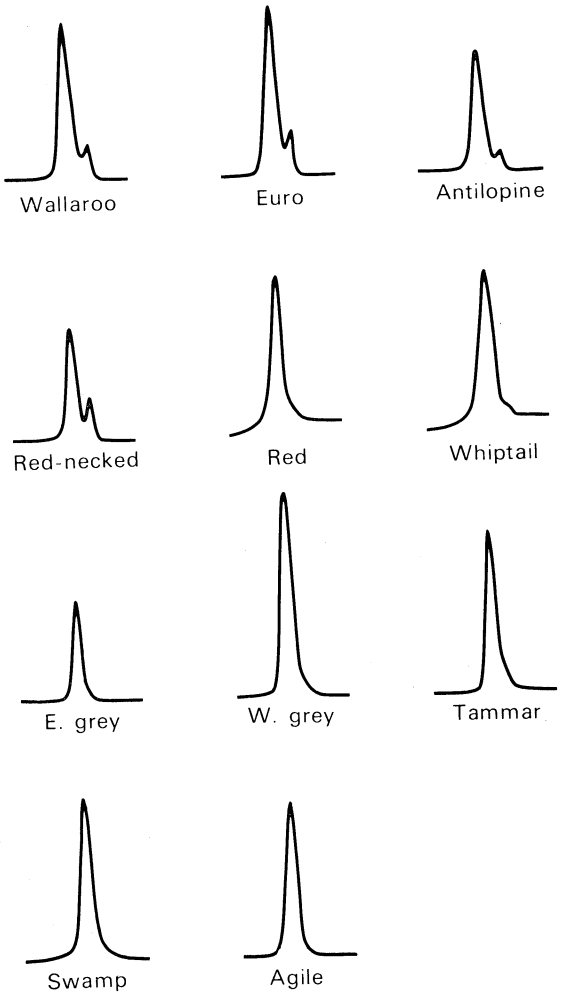


Fig. 1. Equilibrium CsCl density gradients of kangaroo DNAs. Nuclear DNA was extracted from livers and purified by preparative CsCl gradients. Analytical traces of 1–2 μg of DNA from each species are shown; *M. luteus* DNA (ρ = 1·731 g/cm^3) was used as a marker. The main band DNA from each species has a density of 1·698 g/cm^3. Density increases from left to right.

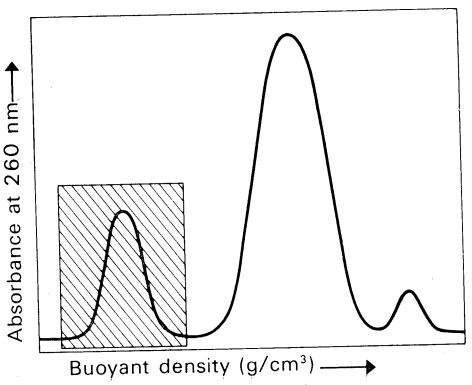


Fig. 2. CsCl buoyant density gradients of red-necked wallaby DNA complexed with actinomycin D. The DNA to actinomycin D ratio was adjusted to 1 : 1, and the density to 1·64 g/cm^3. The hatched peak is the satellite which was isolated and used as a probe.

Choice of Probes

As a result of this survey we decided to isolate the satellite DNAs of the red-necked wallaby and the euro (or wallaroo) in order to use these sequences as probes to investigate the relationships of different species. We also isolated a highly repeated sequence from the heavy shoulder of the red kangaroo DNA peak. In each case, we determined the organization and distribution of the highly repeated sequence in the genome of the species from which it was isolated. We then determined whether similar sequences were present in the genomes of other kangaroo species and compared the sequence and chromosomal organization with that found in the donor species.

The Red-necked Wallaby Probe

The density difference between main band DNA and the heavy satellite of the red-necked wallaby was increased by including the antibiotic actinomycin D into the CsCl gradient (Fig. 2). This technique enabled a simple purification of the satellite DNA which represents about 20% of the chromosomal DNA of the red-necked wallaby (Dunsmuir 1976).

Restriction enzyme analysis showed that the basic repeating unit of the satellite was 2 500 base pairs (bp) long and that the repeat units were arranged in long tandem arrays, the total number of repeats in the genome being approximately 500 000 (Dennis *et al.* 1980). *In situ* hybridization of a radioactively labelled copy of this satellite DNA showed that it originates from the heterochromatic blocks near the centromeres of each of the seven autosomes of the chromosome complement (Dunsmuir 1976).

The Euro Probe

We used as a second sequence probe the satellite DNA that was visible in the neutral CsCl banding of euro (or wallaroo) DNA. In this case, we were able to enhance the separation of the satellite from the remainder of the DNA by using, for the preparative procedure, a Cs_2SO_4 gradient containing silver ions (Venolia and Peacock 1981). Two successive centrifugations gave a good purification of the satellite. In alkaline CsCl gradients it separated into two equimolar bands representing the two polynucleotide chains of the DNA molecules containing the satellite sequence (Venolia 1978). The density difference of the two complementary strands indicates that they differ from each other in their base composition and this in turn suggests that the repeating sequence is likely to be very short. The satellite has a buoyant density of $1 \cdot 710$ g/cm^3, close to that of the red-necked wallaby satellite ($1 \cdot 708$ g/cm^3) but since the satellites do not cross-hybridize they must have quite different nucleotide sequences.

This satellite constitutes approximately 8% of the euro genome, and *in situ* hybridization experiments have shown that it is located in the centromeric heterochromatin of all chromosomes. The satellite from the wallaroo, in all physical and biochemical characteristics examined, was identical to that isolated from the euro.

A number of lines of evidence indicated the presence of considerable sequence heterogeneity in the euro satellite DNA (Venolia 1978) and in order to investigate this more closely, we cloned segments of the satellite molecules using recombinant DNA technology (Fig. 3). These cloned segments were sequenced by direct DNA

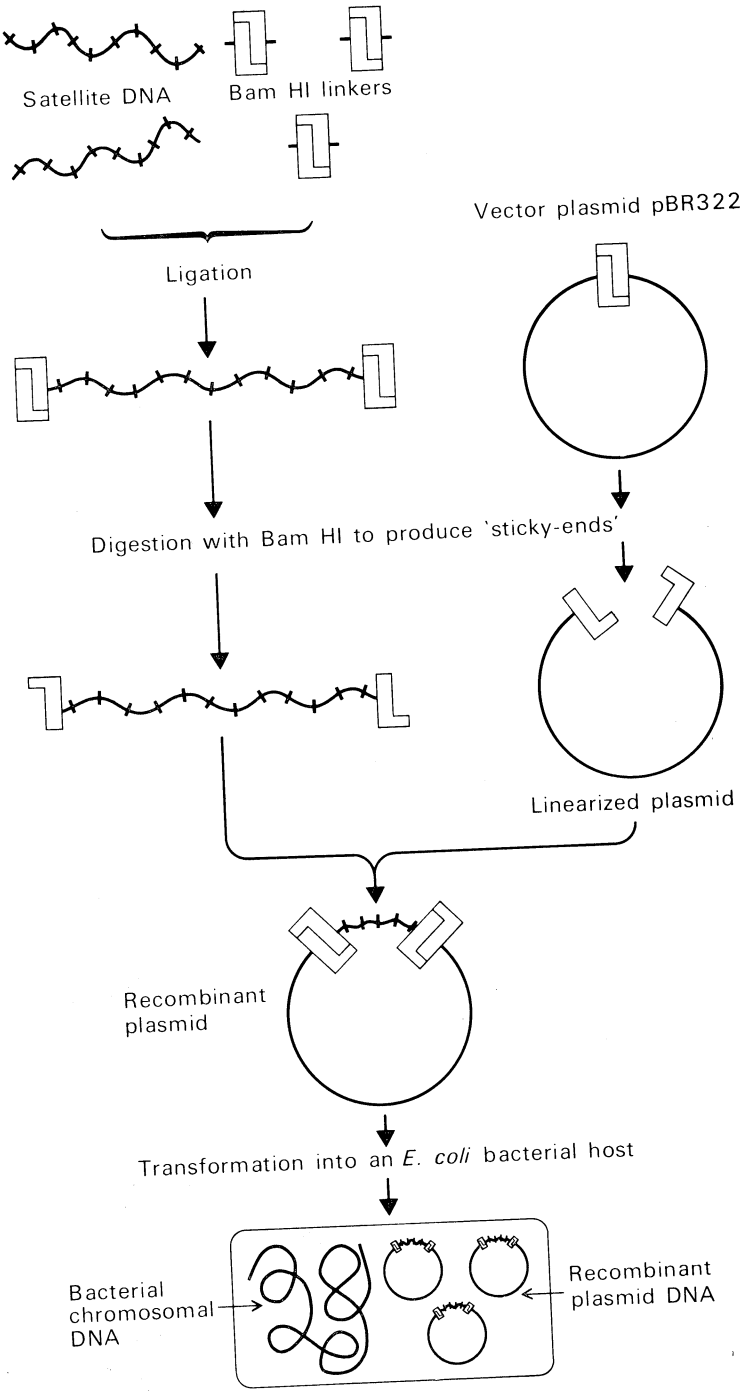
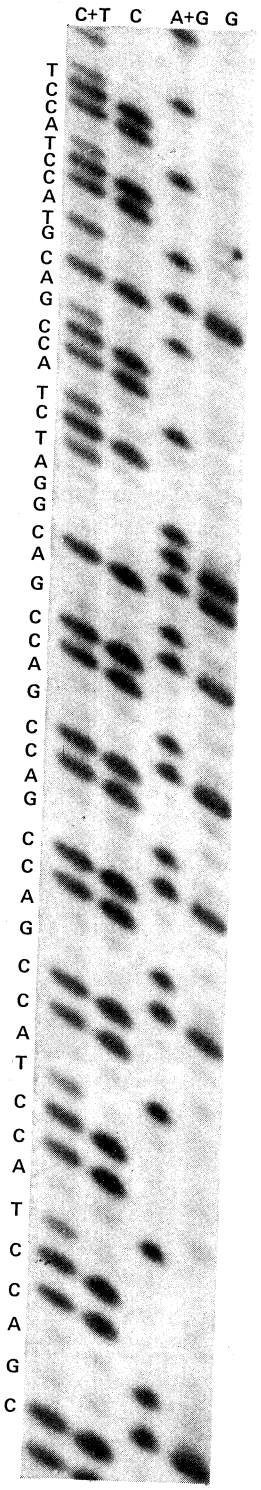


Fig. 3. Cloning of euro satellite DNA. Euro satellite DNA fragments were ligated to BamHI linkers (synthetic decanucleotides containing the BamHI recognition site). After ligation, the satellite with the linkers at its ends was digested by BamHI producing 'sticky ends'. The circular plasmid pBR322 contains one BamHI recognition site, and was linearized by digestion with this enzyme. The euro satellite and the linear plasmid, both with BamHI 'sticky ends' were ligated to form a recombinant plasmid. This plasmid was transformed into an *E. coli* RRI bacterial host.



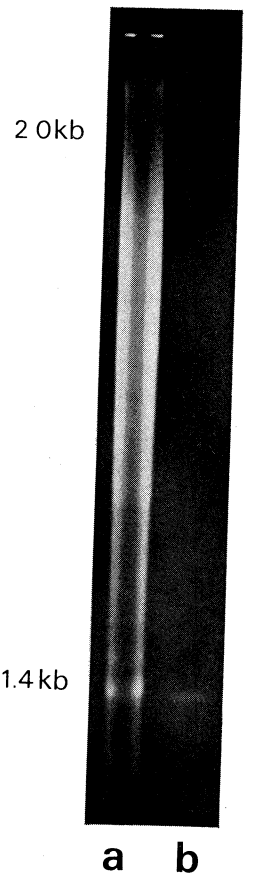


Fig. 4 (*left*). Sequencing gel of cloned euro satellite DNA using the Maxam and Gilbert (1977) sequencing method. The satellite sequence of a clone was cut out with BamHI, 3′-OH ends labelled with [^{32}P]dATP using the Klenow fragment of DNA polymerase I and the two DNA strands separated following alkali denaturation. The four tracks shown in the figure are, respectively, the chemical reactions which cleave the DNA strand at C and T, C only, A and G, and G only. Portion of one of the strands is shown with its nucleotide sequence indicated. The 3′ → 5′ direction is upward in the autoradiogram.

Fig. 5 (*above*). (*a*) Gel electrophoresis of total red kangaroo DNA digested by EcoRI showing a prominent band at 1·4 kb. (*b*) The 1·4 kb band was electro-eluted from the gel. A further electrophoresis showed the recurrent DNA banded at 1·4 kb. This DNA was cloned and used as the highly repeated DNA probe from the red kangaroo.

sequencing methods (Maxam and Gilbert 1977). Two major closely related tetranucleotide repeats (Fig. 4) $^{5'TGGC3'}_{3'ACCG5'}$ (30%) and $^{5'TGGA3'}_{3'ACCT5'}$ (30%) were present, with single base substitutions of these repeats accounting for the remainder of the satellite DNA. All told, there are some 20 million repeats of these tetranucleotide sequences in the genome of the euro.

The Red Kangaroo Probe

The buoyant density profile of red kangaroo DNA (Fig. 1) does not show a distinct satellite but has a heavy shoulder giving an asymmetrical appearance to the DNA peak. When the DNA is analysed in gradients containing either metal ions or antibiotics, we found that there are at least seven different highly repeated sequences (Elizur 1980). These highly repeated sequences have not been isolated in pure form by buoyant density methods, but one repeat class, following its identification by restriction enzyme analysis (Fig. 5), was recovered from the gel and cloned in an *E. coli* plasmid system similar in principle to that described in Fig. 3. This DNA repeat unit of 1400 bp has not been sequenced, but restriction enzyme mapping suggests it has an underlying repeat of 150 bp. Sequencing could provide information on the evolution of the larger unit. When used as a probe for *in situ* hybridization to the chromosomes of the red kangaroo, the 1400 bp unit is found to have major sites on chromosomes 5, 6, 8, 9 and on the X chromosome (Fig. 6). It only accounts for 1–2% of the total chromosomal DNA. Even though the other highly repeated sequences have not been isolated in pure form, *in situ* experiments have established that they too are located primarily in the heterochromatic regions around the centromeres (Elizur 1980).

Patterns of Organization and Change in Highly Repeated DNA

We isolated the three sequence probes in order to use them in analyses which might shed some light on relationships among the various kangaroo species. In each case we have asked questions about representation and organization of the particular sequence in the genome of the species from which it was isolated, and in the genomes of other kangaroo species.

Though a highly repeated DNA sequence is not always useful as an evolutionary probe, a number of investigations have established that closely related species (or taxa) often contain repeat units of the same DNA sequence. Some of the most complete analyses of this type are those in the *melanogaster* subgroup of *Drosophila* species (Barnes *et al.* 1978) and those considering a number of the primates (Gosden et al. 1978). Even though the sequence of the repeat itself may be held constant (Peacock *et al.* 1977), the numbers of repeat units in two related species are sometimes radically different. The rules and mechanisms of change in the numbers of repeats are not fully understood but some evidence points to the involvement of unequal reciprocal exchange between sister chromatids, or even between non-homologous chromosomes, resulting in either increases or decreases in the number of copies at any given chromosomal site (Smith 1973; Carroll and Brown 1976).

Most highly repeated DNA sequences have several locations in the genome (Peacock *et al.* 1977), and for some satellites it is clear that several amplification events have been involved in the generation of the total number of repeats in the genome (Dennis *et al.* 1980).

Relationships Determined using the Red-necked Wallaby Probe

The red-necked wallaby satellite has provided a particularly useful opportunity for examining the temporal and spatial organization of amplification events in a

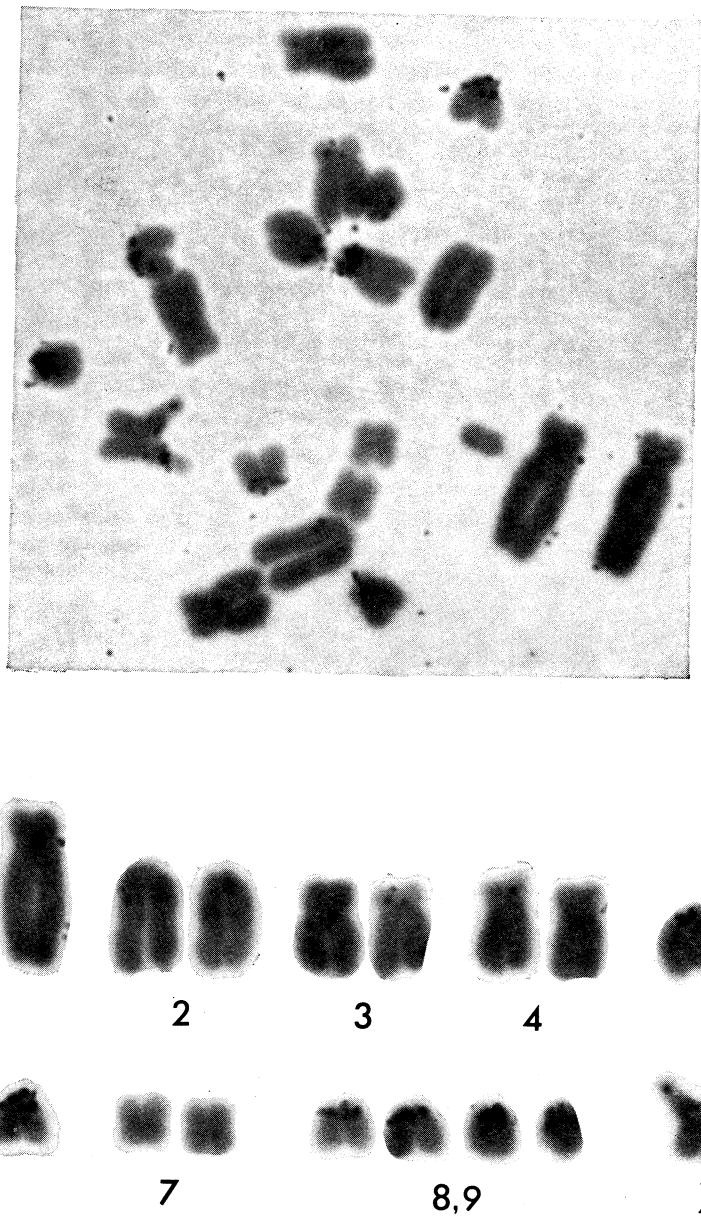


Fig. 6. Autoradiograph of red kangaroo metaphase chromosomes following *in situ* hybridization with [^{3}H]cRNA to the cloned 1·4 kb sequence from the red kangaroo. The chromosomes are numbered according to Hayman and Martin (1974).

satellite DNA. The complex pattern of tandem arrays of different, but closely related, repeats in the genome of the red-necked wallaby (*M. rufogriseus banksianus*) is

exactly the same in the genome of Bennett's wallaby (*M. rufogriseus rufogriseus*) (Dennis *et al.* 1980). Apparently no amplification events have occurred during the past 12000 years, probably the minimum time these two subspecies have been separated.

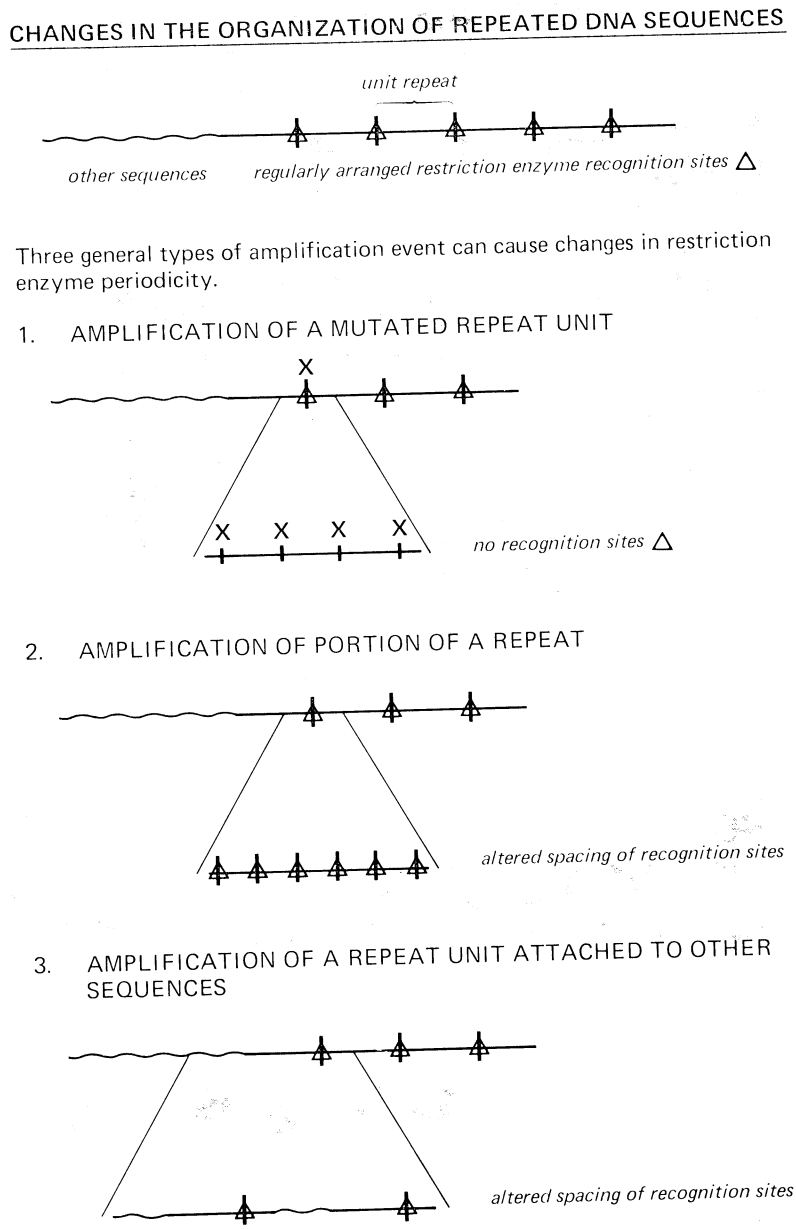


Fig. 7. Diagram showing how changes in repeated DNA sequence organization can lead to altered restriction enzyme periodicity.

The DNA of other kangaroo species was examined to see if any of these repeats could be detected. Substantial numbers were found in the red kangaroo genome

and in the genomes of both the euro and wallaroo. The repeats were also detectable, though in much lower frequencies, probably in the order of 1000 copies or less, in the genomes of the agile wallaby, whiptail wallaby, swamp wallaby, eastern and western grey kangaroos and the tammar (*M. eugenii*).

Apart from the differences in numbers of repeats in different species, there are differences in the nucleotide sequence of the repeating unit. A measure of sequence similarity can be obtained by hybridization of the sequence probe to the genome under investigation. In the case of the red-necked wallaby probe the homologous hybrids with red-necked wallaby DNA were 4°C higher in melting temperature than the hybrids formed between the red-necked wallaby probe and sequences in the DNA of other macropod species. This difference in melting temperature is indicative of approximately 1 in 20 nucleotides in the sequence being changed (Blumenfeld 1973).

Table 1. Melting temperature of heterologous hybrids between [^{3}H]cRNA wallaroo satellite DNA and DNA of other macropodid species

T_m values are accurate to ±1°C. The T_m is the temperature at which 50% of the radioactivity has been released from the filter. Hybridization and melting conditions were as described in Venolia and Peacock (1981)

Species	T_m(°C)	Species	T_m(°C)
M. r. robustus	52·5	*M. eugenii*	47·3
M. r. erubescens	53·0	*M. rufus*	48·4
M. antilopinus	52·5	*M. giganteus*	49·1
M. rufogriseus	49·1	*W. bicolor*	49·1
M. fuliginosus	48·2		

Other differences in the organization of the red-necked wallaby satellite sequence in the various kangaroos are shown by restriction enzyme analyses. In the red kangaroo and in the euro–wallaroo group, the length of the repeating units are different from the 2 500 bp found in the red-necked wallaby. The 2 500 bp unit is detectable in the euro–wallaroo group but these species also have units of 4 500 bp and 9 000 bp (Dennis *et al.* 1980). In the red kangaroo we have not detected any 2 500 bp units but instead find repeats of 3 900 bp. Changes in basic repeat length reflect sequence rearrangements which occur prior to an amplification event (Fig. 7).

Relatedness can be inferred in cases where the repeating unit occurs in identical lengths in two or more taxa, since the amplification event must have occurred in a common ancestor. Units having nucleotide sequence homology but with changed repeat length can also provide clues on the relatedness of species. The failure to find a sequence common to two taxa cannot necessarily be taken as an indicator of lack of relationship, since repeat numbers can be drastically reduced even in a species quite closely related to the reference species. This limitation can be overcome by taking a number of probes into consideration when a relationship pattern between a number of different taxa is being determined.

Our data on frequency of representation of the red-necked wallaby probe in other species have suggested that the red-necked wallaby is more closely related to the red kangaroo and the euro–wallaroo group than has previously been supposed (Richardson and Sharman 1976). Another potential relationship suggested by this probe is between the tammar and the agile wallaby, which have identical organizations

of the sequence in their genomes. Finally, the presence of the sequence in every kangaroo species argues strongly for a monopyhletic origin of the kangaroos.

Relationships Determined using the Euro Probe

The euro probe has confirmed the close relationships within the euro–wallaroo group. The DNAs of wallaroo, eastern and western populations of the euro and antilopine kangaroo all have identical melting profiles in hybridization experiments with the euro probe (Table 1). This means that the nucleotide sequences related to the probe are very similar in these species. Furthermore, *in situ* hybridization shows the distributions of the satellite sequences in the chromosomes of all the taxa are similar (Venolia and Peacock 1981).

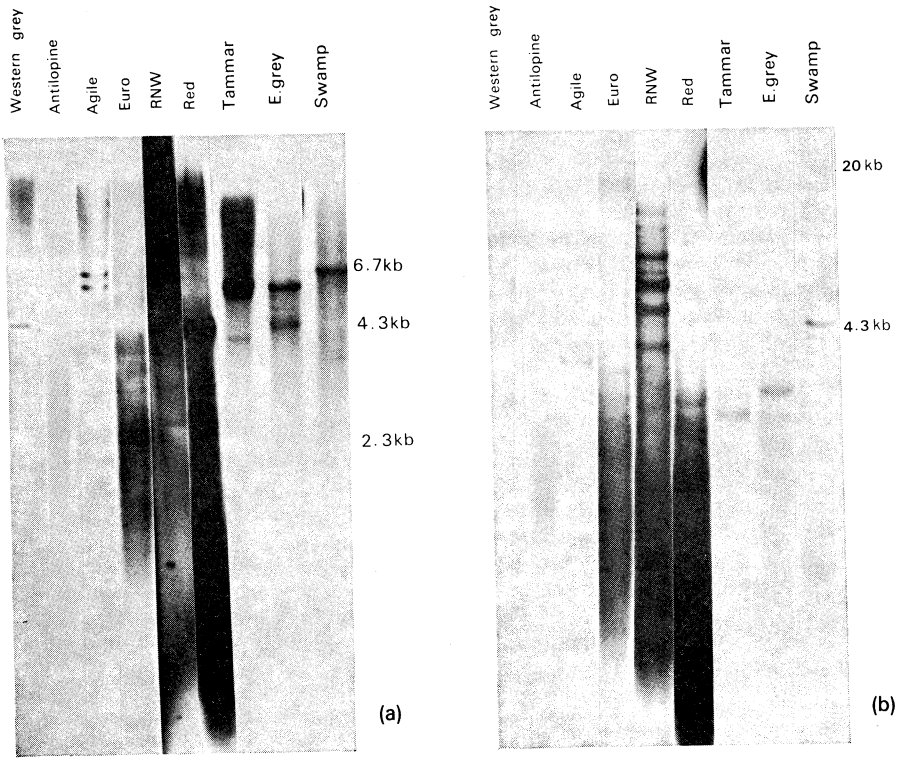


Fig. 8. Organization of sequences complementary to the euro probe in different kangaroo genomes. DNA from each kangaroo species was digested with BamHI (*a*) and Sau3A (*b*) and subjected to electrophoresis in agarose gels. DNA in the gel was transferred to nitrocellulose filters (Southern 1975) and hybridized to ^{32}P-labelled euro probe and autoradiographed. This figure is a composite of different exposure times chosen to yield comparable intensities. It is not a measure of relative amounts of sequence present in the genomes.

This probe also reinforced the conclusion made on the basis of results with the red-necked wallaby sequence probe, that the red-necked wallaby is related to the euro–wallaroo group and to the red kangaroo. There are a significant number of repeats of the euro probe sequence in these other two species whereas it occurs only to a minor extent in other wallabies and kangaroos. There is sequence divergence evident in the repeats present in the red kangaroo and red-necked wallaby and there

are also changes in repeat unit organization. The latter point is shown by hybridization of the probe sequence to chromosomal DNA of a number of macropodid species following digestion with the restriction enzymes BamHI and Sau3A. Figs 8*a* and 8*b* show that in the euro–wallaroo group, most of the satellite sequences have no regularly arranged recognition sites for these two restriction enzymes. The hybridization shows a smear extending over most of the length of the gel, with some

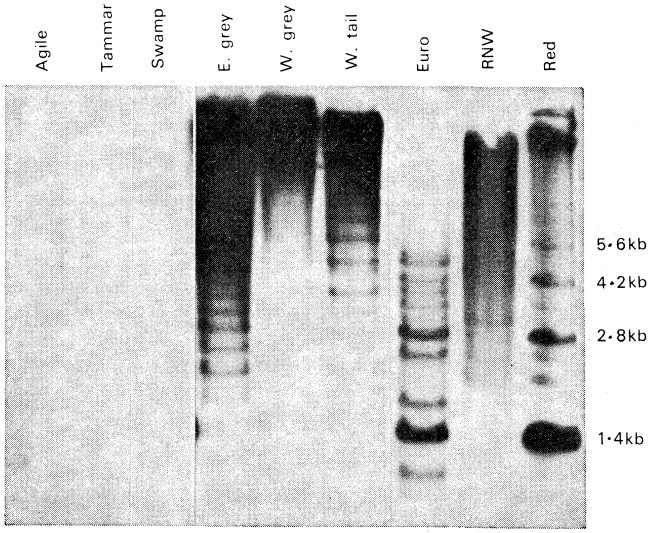


Fig. 9. Organization of sequences complementary to the red kangaroo genomes. DNAs from the different kangaroo species were digested with EcoRI, subjected to electrophoresis, and transferred and hybridized to the red kangaroo probe as described in the legend to Fig. 8. The sequence is present in very low amounts in the swamp wallaby, agile wallaby, tammar wallaby and exposures for these DNAs were approximately 10 times longer than for the other DNAs.

more frequent length classes visible as bands. The existence of the bands indicates that in some parts of the satellite DNA population, there are certain segments which have been generated by amplification of units containing the appropriate recognition sites (Fig. 7). The likelihood of mutational generation of such restriction sites has been demonstrated by direct nucleotide sequencing of cloned segments of the satellite (Fig. 4). The Sau3A site $^{5'GATC3'}_{3'CTAG5'}$ can be generated by a single base change from the prevalent $^{5'TGGATGGA3'}_{3'ACCTACCT5'}$ repeat and the BamHI site $^{5'GGATCC3'}_{3'CCTAGG5'}$ can be derived by two base substitutional changes. These recognition sites must have been generated on many independent occasions to produce the heterogeneous lengths seen following digestion with these enzymes.

In all of the other kangaroo species, the euro satellite sequence is present in regular repeat lengths, shown by distinct bands (Figs 8*a*, 8*b*) of different length in each species.

The difference in band lengths in the eastern and western grey kangaroos is concordant with their recognition as distinct species. The results obtained with the euro probe do not add support to our suggestion that a relationship may exist between the tammar and the agile wallaby since these, like all the other species, have their own distinctive segment lengths.

The simpler patterns of sequence organization seen in the heterologous species may be a consequence of a more restricted chromosomal distribution for the satellite than exists in the euro–wallaroo group. *In situ* hybridization confirmed this in the red-necked wallaby and the red kangaroo, the two species which have representations of the euro sequence sufficient for chromosome mapping. The sequence is not detectable in the autosomes of the red-necked wallaby, but it is present in large blocks on the X and Y chromosomes near the nucleolus organizer (Venolia 1978). It is also present on the X chromosome of the red kangaroo, again near the nucleolus organizer region; the sequence probably accounts for much of the increased size of this X chromosome (Hayman and Martin 1974). The euro satellite also occurs on the four acrocentric chromosomes which have fused in the other species of macropodids (Hayman and Martin 1974; Rofe 1979).

Relationships Determined using the Red Kangaroo Probe

In situ hybridization has shown that the red kangaroo probe is derived from a population of sequences in the heterochromatic blocks of chromosomes 5, 6, 8, 9 and the X chromosome, the major location being on chromosome 5 (Fig. 6). EcoRI restriction of red kangaroo DNA shows two major as well as minor subpopulations (Fig. 9). The 1400 bp band, along with multimer lengths, have major representation (40%), and an equal number of sequences are contained in molecules which are devoid of EcoRI sites, remaining as large molecular weight DNA at the top of the gel. The 1400 bp organizational pattern is also present in the genomes of the euro–wallaroo group, whereas the red-necked wallaby DNA contains only the subpopulation devoid of EcoRI sites (Fig. 9). In this DNA other repeat unit lengths are also detectable. This probe, unlike the euro probe which differentiates the eastern and western grey kangaroos, confirms that these two species are more closely related to each other than to any other species. These two kangaroos have identical arrangements of the red kangaroo sequence, different from that occurring in any of the other kangaroos or wallabies.

The red kangaroo probe has reinforced the suggestion derived from hybridization with the red-necked wallaby probe that the agile wallaby and tammar wallaby are related. The organization of this probe is identical in the two species. Two of the three probes have suggested a close relationship between the agile wallaby and the tammar. Although mammalian taxonomists have not recognized any close relationship between these two species as judged by traditional criteria of morphology, skull morphometrics and behaviour, it is of interest that they cluster closely together in Kirsch's (1977) serological analysis, and they have very similar chromosomes including relative lengths of arms (Hayman and Martin 1974). All three probes suggest that the red-necked wallaby is related to the red kangaroo and the euro–wallaroo group. From the traditional taxonomic viewpoint this is a surprising result.

The probes have confirmed that the eastern and western grey kangaroos are closely related but that their recognition as separate species on the basis of blood protein and other data is warranted.

Sequences related to the three probes were detected in all of the kangaroo species examined and this, as has already been mentioned, emphasizes the single origin of the kangaroos and wallabies. Quite marked differences in sequence and organization of the repeating units were found between species. In discussing these changes, there are few definite time points to apply. In the case of the red-necked and Bennett's

wallabies, the formation of Bass Strait between Tasmania and mainland Australia can be used as the minimum period of subspecific differentiation. This event took place some 12 000 years ago. The separation of the ancestors of the western and eastern grey kangaroos presumably took place during one of the arid phases that were a feature of the fluctuating climate during the Pleistocene. Although few palaeoclimatic data are available, drier conditions than at present are known to have occurred in southern Australia within the last 50 000 years (Walker 1978). It is possible and even likely that speciation in the grey kangaroos took place within this time period. The earliest time point is not nearly so well established, but it seems clear that most of these species have originated subsequent to the times when, from fossil evidence, we know that rather different kangaroos were abundant. The radiation of at least the majority of modern *Macropus* species appears to have taken place during the Pleistocene; they have been in existence no longer than a million years and probably considerably less.

DNA sequence probes have made a contribution to an understanding of relationships of the macropodids and it seems probable that they will also be useful in examining the relationships of the macropodids to the other major groupings of marsupial species.

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